Review

Axon guidance and synaptic maintenance: preclinical markers for neurodegenerative disease and therapeutics

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Axon-guidance-pathway molecules are involved in connectivity and repair throughout life (beyond guiding brain wiring during fetal development). One study found that variations (single-nucleotide polymorphisms [SNPs]) in axon-guidance-pathway genes were predictive of three Parkinson's disease (PD) outcomes (susceptibility, survival free of PD and age at onset of PD) in genome-wide association (GWA) datasets. The axon-guidance-pathway genes *DCC*, *EPHB1*, *NTNG1*, *SEMA5A* and *SLIT3* were represented by SNPs predicting PD outcomes. Beyond GWA analyses, we also present relevant neurobiological roles of these axon-guidance-pathway molecules and consider mechanisms by which abnormal axon-guidance-molecule signaling can cause loss of connectivity and, ultimately, PD. Novel drugs and treatments could emerge from this new understanding.

Introduction

Brain function is based on precise neuronal-network formation during development, which is largely controlled by attractive and repulsive axon-guidance molecules [1]. The spatially and temporally regulated expression of the guidance cues navigates the outgrowth of axons and specifies their termination zones and synaptic partners [2,3]. Many guidance molecules persist in the adult central nervous system and extensive studies have shown that these factors have roles in maintenance and plasticity of neural circuits [4]. Such factors also participate in adult brain repair and regeneration after brain injury [4]. Emerging evidence indicates that guidance molecules could also have important roles in neurodegenerative disorders owing to altered signaling during development and/or owing to altered maintenance of synaptic connections in the adult. There is some neuropathological evidence of misrouted fibers in the adult human brain, and in Parkinson disease (PD) and Alzheimer's disease [5].

PD is primarily a movement disorder characterized by resting tremor, bradykinesia, rigidity and postural instability. The pathological underpinnings of these motor symptoms include a marked degeneration of dopamine (DA) neurons in the substantia nigra (SN), with resultant striatal DA deficiency. The lifetime risk for PD in the general population is 2% [6] and the incidence of PD rises steeply with age [7]. Presently, there is no method of preventing PD or of halting its progression. However, a method of predicting PD was recently reported [8]. This genomic pathway approach involved measuring common variations (single-nucleotide polymorphisms [SNPs]) within a family of genes that encode the wiring of the brain during fetal development and that encode the maintenance and repair of brain wiring throughout life (the axon-guidance pathway). The additive effects of these variants (SNP models) were highly predictive of PD susceptibility, survival free of PD and age at onset of PD in two independent genome-wide association (GWA) datasets. Several axon-guidance-pathway genes represented by SNPs in the predictive models were differentially expressed in a genome-wide expression profiling dataset. SNP models for PD were subsequently refined and compared with axon-guidance-pathway SNP models that were highly predictive of amyotrophic lateral sclerosis (ALS) [9]. Distinct gene signatures for the two diseases were defined within the pathway, and ultimately these diseases could be predicted with >90% sensitivity and specificity. However, the findings for the axon-guidance pathway and ALS await validation in a second GWA dataset.

Here, we summarize axon-guidance-pathway SNP models for PD outcomes in two GWA datasets and also describe the roles and mechanisms involved in the actions of these specific axon-guidance-pathway genes or gene products as candidate targets for neuroprotective therapies. We also summarize experimental data for PD, relevant DA neurons and their connected target neurons as rationales for these therapeutic targets.

Axon-guidance-pathway SNP models nominate therapeutic targets for PD

A GWA study of PD mapped the semaphorin 5A (*SEMA5A*) gene as a susceptibility locus for PD [10] and, thus, high-

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lighted a possible role for axon guidance in the pathogenesis of PD. Although disease associations for single SNPs have small effects and are difficult to replicate, additive effects of SNPs within functionally related genes could have large effects and replicate across studies.

We have summarized the convergence of results for the predictive SNP models for recorded clinical outcomes in two GWA datasets (Table 1). There are five axon-guidance-pathway genes that were represented by SNPs in all of the models: deleted in colorectal carcinoma (*DCC*), ephrin receptor B1 (*EPHB1*), netrin-G1 (*NTNG1*), *SEMA5A* and *SLIT3*. However, it is noteworthy that four of the five genes did not have informative probe sets (not expressed) and one gene (*EPHB1*) was expressed similarly in PD cases and controls (Table 1). This raises the intriguing possibility that the mechanism by which these genes contribute to PD is temporally remote from the clinical terminal state, such as early degenerative changes in the years preceding diagnosis and possibly even during brain development (this is called the miswiring hypothesis).

Although such a selection of candidate targets is based on the convergence of final SNP models within two independent samples, several models of SNPs predicted PD outcomes in those samples [8,9]. However, it should be pointed out that Li *et al.* [11] could not replicate such conclusions from their sample data, possibly because different axon-guidance-pathway SNP models will be predictive of PD in other samples and not the final models highlighted (i.e. locus heterogeneity) [11]. By contrast, two other studies performed pathway-based analyses of three GWA datasets of PD combined and concluded that axon guidance was significantly associated with PD susceptibility and ranked at or near the top of several hundred pathways in two of the three datasets [12,13]. In the following description, we illustrate possible mechanisms by which variability in axon-guidance-pathway genes might contribute to the pathogenesis of PD.

Neurobiological basis of axon-guidance pathways as therapeutic targets for PD

The axon-guidance pathway consists of four major classes of ligands (ephrin, netrin, semaphorin and slit proteins), their respective receptors (e.g. eph, DCC and unc, neuropilin and plexin, and robo proteins) and several downstream signaling proteins. Together, these chemical signals provide a series of attraction and repulsion cues that direct axons to their targets. A diagram of these processes can be found in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/pathway/hsa/hsa04360.html). For each major class of ligands and receptors, axon-guidance-pathway SNP-based models nominated a therapeutic target (Table 1) and each such molecule is discussed in a neurobiological context later.

Netrin

Secreted netrins (netrin-1 to netrin-4) and their receptors are one of the well-characterized axon-guidance-pathway families [14]. Netrins can bind to DCC or Unc6/Unc5 receptors [15], exhibiting attractive and repulsive activities in the axon guidance, respectively. In the midbrain DA system, netrin-1 protein enhances DA axonal outgrowth and functions as an attractant of DA axons. Such effects were mediated by the DCC receptor as demonstrated by antibody blockade [16]. The association of this ligand–receptor family to the pathogenesis of PD has been modeled in Netrin-1-null mice, which display a loss of DA neurons and mislocalized and mistargeted DA neurons in the nigrostriatal tract during development[*]. DCC-deficient adult mice show altered DA transmission and locomotor activity accompanied by reduced dendritic-spine density in the cerebral cortex [17]. These findings demonstrate that DCC is a crucial molecule in the development of DA circuitry formation, and alterations in the *DCC* levels can lead to cognitive and behavioral abnormalities in the adulthood. Netrin-1 also defines the structural organization of the striatum, which determines the synaptic formation of DA afferents [18]. In addition to its role in the neuronal connectivity, netrin-1, DCC and UNC5 also mediate neuronal survival that involves the PIKE-L-stimulated phosphatidylinositol 3-kinase cascade [19]. Some SNP models indicate that variability in the *DCC* gene, and possibly in *UNC5*-encoding genes, predicts PD outcomes (Table 1). A potential outcome of altered function of secreted netrin-1 and its receptors, which would possibly disorganize synaptic circuitry and alter DA transmission, would then increase susceptibility for individuals to develop PD.

Netrin-G1 and netrin-G2 (NTNGs) are glycosyl-phosphatidylinositol-anchored membrane proteins [20,21]. Unlike netrin-1, they are not secreted ligands but anchored ligands and they do not bind to the DCC or Unc5 receptors but to netrin-G ligands (NGLs) [21,22]. This subgroup of netrin proteins contains multiple alternatively spliced isoforms that are expressed in the developing brain and in the adult brain [20,21,23]. Lack of orthologs of these genes in *Caenorhabditis elegans* or *Drosophila melanogaster* indicates a specific role in the brain function of vertebrates. Mutations in the *NTNG1* gene cause an atypical presentation of Rett Syndrome with epileptic seizures of early onset [24,25]. Postmortem studies have demonstrated differential expression of *NTNG1* in schizophrenia [26,27]. Rett syndrome, epilepsy and schizophrenia are disorders attributed to impaired synaptic formation, functioning and plasticity within glutamate and γ-aminobutyric acid (GABA)-transmitting neurons [28–31]. Indeed, experiments demonstrate that NTNG–NGL ligand–receptor pairs function as synaptic cell-adhesion molecules that regulate synapse formation and maintenance [20,21,32]. NGLs contain an intracellular PDZ (postsynaptic density [PSD]-95/disc large/zona occludens-1)-binding domain that interacts with the PSD-95 family of proteins to modulate the formation of excitatory synapses [22]. Overexpression of NGL2 increases the number of PSD-95-positive dendritic protrusions, and NGL2 aggregation leads to clustering of postsynaptic proteins including NR2A (NMDA receptors), indicating that NTNG–NGL adhesion proteins partner with PSD-95 to recruit PSD-95-associated postsynaptic proteins for excitatory synapse differentiation [22]. By

[*] Li, J. *et al.* (2007) Netrin 1 and Slit 1/2 are required for correct localization of midbrain dopaminergic cell bodies and targeting of their axons [abstract] *Soc. Neurosci.* 460.4/E36.

Table 1. Classic axon-guidance molecules associate with genetic risk for PD[a]

	GWA data (from Ref. [10])			GWA data (from Ref. [69])			Expression data (from Ref. [70])		
Gene	**Susceptibility**	**Survival**	**Age at onset**	**Susceptibility**	**Survival**	**Age at onset**	**Substantia nigra**	**Putamen**	**Caudate**
ABL1			X[b]						
ABLIM2		X	X	*		X			
CDC42	X		X	X					
CHP	X								
CXCR4			X						
DCC	X	X	X	X	X	X			
DPYSL2		X	X		X	X			
EFNA5	X		X	X	*	X			
EPHA4	X		X	X					
EPHA8		X							
EPHB1	X	X	X	X	X	X			
EPHB2	X			X	*	*			
FYN	X			X					
GNAI3	X								
GSK3B	X			X					
KRAS		X							
MRAS	X								
NFATC2		X	X		X	X			
NFATC4		X	X						
NTNG1	X	X	X	*	X	X			
PAK1			X						
PAK3		X	X			X			
PAK4	X								
PAK6		X	X						
PAK7	X		X	X	*	X			
PLXNA2	X			X					
PLXNC1	X	X	X	X	X				
PPP3CA	X	X	X	X	X				
RAC2	X			X					
ROBO1		X	X	*	X	X			
ROBO2		X	X	*	X	X			
ROCK2		X			X	*			
RRAS2	X	X		X					
SEMA3A		X		*					
SEMA3D		X							
SEMA3E		X	X	*	X	X			
SEMA4D			X			X			
SEMA5A	X	X	X	X	X	X			
SLIT2			X	*	*	X			
SLIT3	X	X	X	X	X	X			
SRGAP1		X	X	*					
SRGAP3		X	X	*	X	X			
UNC5A		X							
UNC5C	X			X	*	*			
UNC5D		X	X						

[a]Summary data for final SNP models (PD susceptibility, survival free of PD and age at onset of PD) in two GWA datasets (primary GWA dataset [10] and secondary GWA dataset [69]), and summary data for a genome-wide expression profiling dataset [70]. The 45 genes listed are a subset of 128 axon-guidance-pathway genes annotated by KEGG and are included because they were represented by at least one SNP in the final models for at least one outcome in a primary GWA dataset. For the GWA data, an X is used to indicate that a given gene was represented by at least one SNP within a given final model in the primary GWA dataset or that a given gene was represented by at least one SNP within a given final model in the secondary GWA dataset, when those SNP models were restricted to the 45 genes identified in the primary GWA dataset [10,69]. The asterisk (*) indicates that a given gene was otherwise represented by at least one SNP within a given final model in the secondary GWA dataset, when those SNP models were not restricted to the 45 genes identified in the primary GWA dataset (SNPs from as many as 128 axon-guidance-pathway genes, as annotated in KEGG, were eligible to be included in the final models for our unrestricted analyses of the secondary GWA dataset) [69]. Highlighted in yellow are the five genes that were represented by at least one SNP in each of the final models for the primary GWA dataset and in each of the final models for the secondary GWA dataset (restricted or unrestricted analyses). For the expression data, the cells are clear when the gene had informative probe sets but was not differentially expressed in cases and controls; shaded gray when there were no informative probe sets for the gene; red when the gene was overexpressed in PD cases versus controls; green when the gene was expressed less in PD cases versus controls.

contrast, NGL2 inhibition or NGL2 silencing via small interfering RNA reduces the number of excitatory synapses and current potentials [22]. It is known that NGL1 is abundantly expressed in the striatum and that glutamatergic synapses are selectively depleted in PD models [22,23,33]. Some SNP models indicate that variability in the *NTNG1* gene predicts PD outcomes (Table 1). Given these genetic and biological findings, it is possible that differential expression of *NTNG1* alters dopaminergic and glutamatergic circuitry and, thus, contributes to pathogenesis of PD (Figure 1). However, such mechanisms of developmental (i.e. miswiring) or lifelong (i.e. impaired

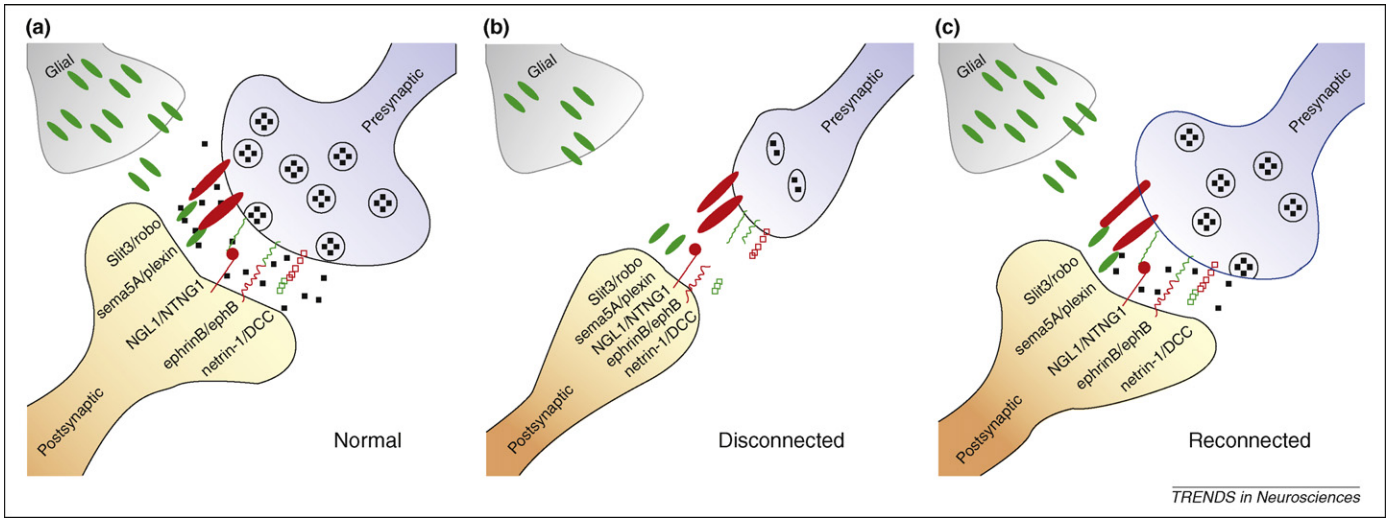

Figure 1. Axon-guidance molecules: ligands and receptors on pre- and post-connective elements in neural circuitry. The drawing shows a simplified model of synaptic disconnection and repair associated with axon-guidance molecules (axon in blue; dendrite in yellow; glia in gray). **(a)** Axon-guidance molecules such as the netrin-G1 (NTNG1), sema5A (SEMA5A) and Slit3 (SLIT3) ligands (and their respective receptors) and the DCC and EphB1 (EPHB1) receptors (and their respective ligands) contribute to the modulation and maintenance of synaptic transmission. **(b)** Genetic variation in these axon-guidance-pathway cues might result in abnormal synaptic connections in the developing brain or in normally connected synapses that are more vulnerable to endogenous or exogenous toxins that trigger synaptic dysfunction and cell death. **(c)** Synapses can be reconstructed or repaired either by transplanting new cells or by modifying the expression of these axon-guidance-pathway cues.

axon maintenance, repair and synaptic plasticity) structural changes require further study.

Slit

Expression of slits and robos (also known as 'roundabout') occurs in concert with the development of midbrain DA neuronal pathway formation. Their complementary regional distribution of slits and robos indicate both structural and functional roles in nigrostriatal and striatonigral pathways [34]. Indeed, it has been shown that loss of Slit1 and Slit2 expression results in an abnormal course of the nigrostriatal pathway through the diencephalon [35]. These mutant mice also exhibit errors in the projection of the nigrostriatal pathway in the striatum*. Whether DA axonal mistargting caused by *SLIT1* and/or *SLIT2* gene deficiency during development will lead to aberrant DA function in the adult remains to be determined. One set of bioinformatic analyses of SNPs indicated that variability in the *SLIT3* gene, and possibly in other *SLIT* and *ROBO* genes, predicts PD outcomes (Table 1). *SLIT3* mRNA expression is absent in the embryonic striatum but abundant in the adult striatum [34]. *SLIT3* knockout influences diaphragm and kidney development but no brain deficits have been reported [36]. However, Slit3 expression is upregulated after spinal cord injury and might be inhibitory for the regenerating axons [37]. The secretion and function of Slit3 is largely unknown, particularly in the DA system. Nonetheless, it has been reported that Slit3 is localized in the mitochondria and its expression can be induced by lipopolysaccharide (LPS) [38]. As a result, Slit3 stimulates cell motility of macrophages, indicating that Slit3 is involved in a LPS-induced inflammatory response that could contribute to PD pathogenesis [39]. The Slit/robo pathway also mediates signals of cell degeneration and tissue remodeling [40]. Given these functional roles of Slit3, it is possible that the Slit3/robo pathway might initiate and accelerate PD processes or progression.

Ephrin

In *EPHB1*-knockout mice, there is a significant cell loss in the SN pars reticulata, but there is no obvious change in the number of DA neurons in the SN pars compacta [41]. The mice displayed spontaneous locomotor hyperactivity [41]. It has also been noted that *EPHA5* (EPH receptor A5)-knockout mice developed neurochemical and behavioral deficits including impaired striatal function, as assessed by an active-avoidance paradigm [42]. These observations indicate a role for ephrin/eph signaling in the structure and connectivity of the dopaminergic pathway. However, this signaling is likely to involve multiple members from the EphA and EphB receptor subfamilies with many potential interactions. Because the ephrin/eph family has a variety of important functions including axonal outgrowth and pruning, neuronal connectivity, synaptic maturation and plasticity, and neuronal apoptosis [2,43–45] it is plausible that variability in such molecules could contribute to the initiation and progression of neurodegenerative diseases. Several SNP models indicate that variability in the *EPHB1* gene, and possibly in other *EPH*-ligand and *EPH*-receptor genes, predicts PD outcomes (Table 1). In addition, it was recently suggested that mutations in the vesicle-associated membrane protein B (*VAPB*) gene cause ALS in families via the ligand-binding effects of the miscoded protein on its eph receptors [46]. Axon-guidance-pathway models for ALS pathology include SNPs in several ephrin and eph receptor genes, and the *EFNA5* and *EPHB1* genes are represented by SNPs in final models for all three outcomes proposed in one study to date (ALS susceptibility, survival free of ALS and age at onset of ALS) [9]. However, the findings for axon-guidance-pathway SNP models in ALS require validation using individual-level genotyping data from other GWA study datasets (as yet, not publicly available).

Semaphorin

Systematic meta-analysis has confirmed that variability in the plexin A2 (*PLXNA2*) gene is associated with

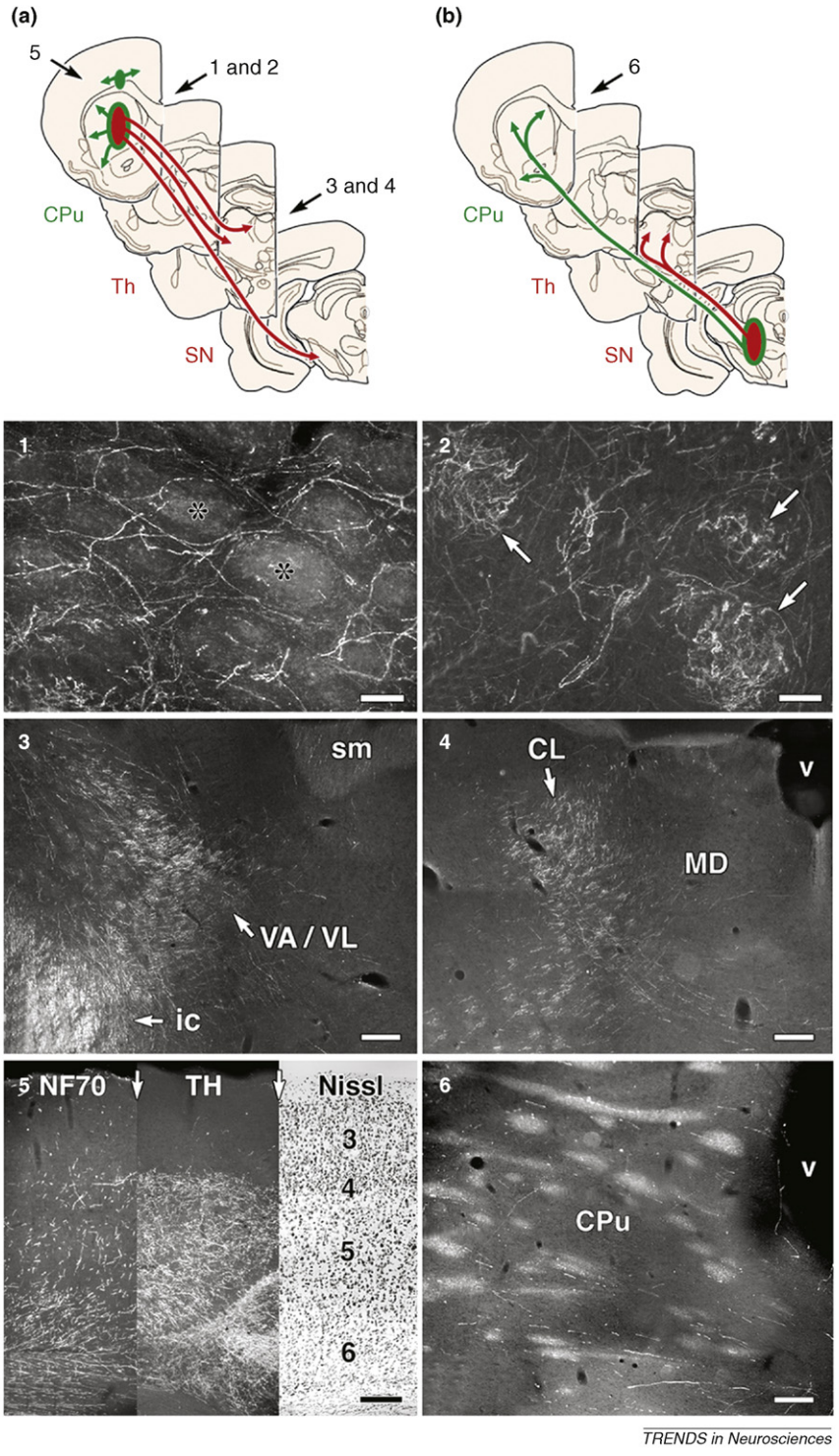

Figure 2. Evidence of selective and appropriate neuro- and chemotropism in the normal adult brain. Drawings and photomicrographs illustrating axonal-growth patterns from cross-species fetal ventral midbrain cell transplants into adult rats. Diagrams **(a)** and **(b)** summarize axonal-growth patterns to specific rat-brain targets from placements in the rat caudate putamen (a) or ventral midbrain (b). Tyrosine hydroxylase (TH)-positive axonal projections are diagrammed in green and non-TH projections are in red. Photomicrographed axons are TH-positive in 1 and 5, but otherwise are NF70 positive. TH-positive axons grew into the gray matter of the adjacent host striatum (1) but avoided corticofugal fiber bundles (asterisks). NF70-positive axons grew axons into both gray and white matter (2), with a tendency to concentrate in fiber bundles (arrows). Remarkably, NF70-positive axons innervated the appropriate thalamic nuclei from transplants in either rostral (a) or caudal (b) positions (see photomicrographs 3 and 4), but TH-positive donor axons were not observed within the thalamus. Three adjacent cortical sections through the parietal cortex (5) show that NF70-positive and TH-positive axons from transplants placed in the striatum densely ramified within the normal target zones of lower cortical layer (5 and 6), but formed a sharp boundary at layer 4 (Nissl staining shown for laminar comparisons), indicating the presence of relevant axon-guidance cues also in the adult brain. Axons from transplanted sites in the midbrain grew rostrally to innervate the striatum in 6 (NF70-positive axons shown). These data clearly illustrate by using a transplant bioassay that growing axons register sufficient axon-guidance and growth-cone signals in the adult brain to reconnect pathways precisely to those of normal developmental target selection. Scale bars: 50 μm (1, 2) and 200 μm (3–6). Abbreviations: CL, centrolateral thalamic nucleus; CPu, caudate putamen; ic, internal capsule; MD, mediodorsal thalamic nucleus; sm, stria medularis; SN, substantia nigra; Th, tyrosine hydroxylase; VA/VL, ventroanterior/ventrolateral thalamic nuclei; V, ventricle. Figure adapted, with permission, from Ref. [68].

susceptibility to schizophrenia, another disorder characterized by aberrant DA transmission [47]. One set of SNP models indicates that variability in the *SEMA5A* gene, and possibly in other *SEMA*-ligand and *PLXN*-receptor genes, also predicts PD outcomes (Table 1). However, some new groups have challenged the genetic association of *SEMA5A* with PD risk. Clarimon *et al.* [48] conducted a gene-association study in two independent case-control series of patients from Finland and Taiwan. They found in the Taiwanese, but not in the Finnish, cohort an associated risk of *SEMA5A* in the locus as reported by Maraganore *et al.* [10] in patients from Minnesota. Another group [49] could not confirm that *SEMA5A* is a risk-conferring gene in a Polish and an Asian population. These discrepancies might have resulted from the use of different cohorts and genetic heterogeneity. Although that is interesting in itself, and probably reflects different genetic variations in separate populations (and genetic maps and/or methods used), independent replication across populations should be conducted to clarify the role of carrying SNP variation in this gene and the risk for developing PD. How could sema5A increase susceptibility of PD? Sema5A is a membrane-bound protein and interacts with receptor of plexin B3 [50]. *Sema5A*-null mice die at E11.5 and E12.5 owing to defects in cranial vascular system and blood vessels [51]. It is known that sema5A is expressed in the cerebral cortex, basal ganglia, thalamus and other regions in rat brain [52]. During development, sema5A functions as an attractive and repulsive guidance molecule, and altered expression has been linked with aberrant development of axonal connections in the forebrain [53,54]. In the adult brain, it has been shown that sema5A expression in the glial cells inhibits axon growth by retinal ganglion cells after injury [55]. Furthermore, it has been shown that haploinsufficiency for sema5A is involved in mental retardation in Cri-du-chat, a disorder caused by deletions of chromosome 5p where sema5A resides [56]. Another genetic study of autism indicates that sema5A is a candidate gene in the etiology of idiopathic autism in which synaptic dysfunction of specific neurons play a part in the disease development [57]. Importantly, it has been known that several members of the semaphorin family participate in various phases of immune responses, from initiation to terminal inflammatory processes, and therefore are also called 'immune semaphorins' [58]. Sema5A is one of the molecules that interact with forebrain embryonic zinc finger-like (FEZL), and that interaction induces genes related with immune response including tumor necrosis factor-α and interleukin-8 expression. The FEZL–sema5A pathway increases susceptibility of cow to mastitis [59]. Overall, these studies indicate that sema5A exhibits multiple functions in axonal connection and regeneration, vascular development and the immune system. Altered expression of sema5A is associated with some brain disorders and infectious diseases. These data also provide reasons to examine how the variability of sema5A could be involved in increasing the risk of PD by causing dysfunction of synaptic activity and inflammation. The latter can sensitize the response of DA neurons to endogenous and exogenous insults [39,60].

Can new therapies be accomplished by modifying structural connectivity and growth processes in the adult brain?

In summary, using genomic pathway analyses and available neurobiological and medical data, several molecules involved in axon-guidance-pathway formation seem to be relevant to PD [8]. Such genetic variability in the axon-guidance pathway might result in developmental defects in brain wiring [35,61,62] and/or in lifelong defects in axon maintenance, repair and synergistic connectivity [63,64], which could contribute to the pathogenesis and dysfunction seen in PD. Given that directional guidance cues persist in the adult brain (as evidenced by target seeking of axons of transplanted fetal cells to distant and specific adult host neurons; Figure 2), chemorepulsive and chemoattractive molecules are clearly present in the adult brain [65,66]. Furthermore, mechanistic studies to define how variations in genes such as *DCC*, *EPHB1*, *NTNG1*, *SEMA5A* and *SLIT3* predispose to PD could further expand an understanding of PD and other degenerative brain disorders. In the future, connectivity-controlling molecules could be targeted therapeutically for the purposes of enhancing reconnection or repairing neuronal pathways [66–68].

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